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Occurrence of Bacterial Spot in Illinois Tomato Fields and Characteristics of the Causal Agents

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***Abstract*. Bacterial spot, caused by *Xanthomonas* spp., is one of the most important diseases of tomato in Illinois. Field surveys were conducted during 2017–19 to assess occurrence of bacterial spot in commercial tomato fields. Severity of foliage and fruit infection was recorded, and symptomatic samples were collected from three-to-five cultivars in three different farms in each of northern, central, and southern regions of Illinois. Severity of symptomatic foliage ranged from 0% to 91% (average 36.7%) and incidence of symptomatic fruit ranges from 0% to 30% (average 10.8%). During the surveys, 266 *Xanthomonas* isolates were collected and identified as *Xanthomonas gardneri* and *X. perforans* using *Xanthomonas*-specific *hrp* primers. Eighty-six percent of the isolates from the northern region were identified as *X. gardneri*, whereas 73% of the isolates from southern region were identified as *X. perforans*. Isolates from the central region were identified as *X. perforans* and *X. gardneri* 53% and 47% of the time, respectively. Multilocus sequence analysis using six housekeeping genes (*fusA*, *gap-1*, *gltA*, *gyrB*, *lepA*, and *lacF*) revealed the endemic population of *X. gardneri* and *X. perforans*. In addition to *Xanthomonas,* nine non-*Xanthomonas* bacterial genera were isolated from the samples, with most of the isolates classified as *Microbacterium*, *Pantoea*, and *Pseudomonas*.**

Bacterial spot of tomato (*Solanum lycopersicum* L.) was first identified in South Africa (Doidge, 1921). Originally, bacterial spot was thought to be caused by only one species, *Xanthomonas campestris* pv. *vesicatoria* (Stall et al., 1994; Vauterin et al., 1995); however, subsequent studies divided it into two species: *X. axonopodis* pv. *vesicatoria* (group A) and *X. vesicatoria* (group B) (Vauterin et al., 1995). Currently, the following species are considered to be the casual agents of the bacterial spot disease complex of tomato: *X. euvesicatoria* (group A), *X. vesicatoria* (group B), *X. perforans* (group C), and *X. gardneri* (group D) (Jones et al., 2000, 2004).

Bacterial spot of tomato is characterized by necrotic lesions on leaves, stems, flowers, and fruits (Egel et al., 2018; Jones, 1991). During the initial stages of disease development, symptoms develop as circular water-soaked lesions that dry out to give a greasy appearance, and eventually turn dark-brown to black (Jones, 1991). Some species-specific symptoms include shot-hole type lesions caused by *X. perforans* and lesions with a more water-soaked appearance caused by *X. gardneri* (Stall et al., 2009). *X. euvesicatoria* and *X. vesicatoria* have been generally associated with lesion development on fruits, though recent publications have also shown *X. gardneri* causing large, deep fruit lesions (Ma et al., 2011). Generally, the disease is favored by warmer and more humid conditions for progression and spread (Araujo et al., 2010); however, *X. gardneri* appears more often in cooler temperatures (Jones et al., 1988) and causes more severe disease at 20 °C than the other three species (Araujo et al., 2010). *Xanthomonas* bacteria are disseminated within a field by wind-driven rain and by mechanical means such as grafting, clipping, tying, harvesting, and spraying pesticides (Lindeman and Upper, 1985; McInnes et al., 1988).

Researchers have used diagnostic methods for identification of bacterial species, which are based on the *hrp* gene clusters that are highly conserved among several phytopathogenic bacteria (Fenselau et al., 1992; Hwang et al., 1992). Leite et al. (1994) was the first to use *hrp* gene clusters as a diagnostic tool for identification of species and pathovars of incitants of bacterial spot. Fragments of different *hrp* genes were amplified using a polymerase chain reaction (PCR) assay, followed by restriction digestion using endonucleases to allow detection of 28 different *X. campestris* pathovars. Obradovic et al. (2004) developed specific PCR primers that amplify a 420 base pair (bp) fragment of *hrpB7* from four bacterial spot pathogens causing disease in tomato. This diagnostic method is still used in routine diagnostic tests.

Genotypic characterization of prokaryotes can be performed using multilocus sequence analysis (MLSA), which differentiates between bacterial strains using a small number of allelic mismatches found in housekeeping genes (Maiden et al., 1998). Six housekeeping genes (*fusA*, *gap-1*, *gtlA*, *gyrB*, *lacF*, and *lepA*) were used to create a MLSA database of *Xanthomonas* strains (Almeida et al., 2010).

The objectives of this study were to 1) assess the incidence and severity of bacterial spot disease of tomato in Illinois; 2) identify the *Xanthomonas* spp. causing the bacterial spot disease; 3) determine the genetic diversity among *Xanthomonas* isolates and examine the phylogenetic relationships using housekeeping genes; and 4) identify the non-*Xanthomonas* bacteria associated with bacterial spot caused by *Xanthomonas* spp.

Materials and Methods

Field surveys. In 2017, 2018, and 2019, field surveys were conducted to assess the severity of bacterial spot disease on tomato cultivars grown in Illinois. Each year, commercial tomato farms located in the northern, central, and southern regions of Illinois (Fig. 1) were visited three or four times throughout the growing seasons (Table 1). A total of 13 different tomato cultivars were evaluated, including Biltmore, Brandywine, Carolina Gold, Chef's Choice, Dixie Red, Heirloom, Jolene, Phoenix, Primo Red, Pony Express Plum, Red Deuce, Red Morning, and Rocky Top.

At each tomato field, 10 randomly selected plants from each cultivar were evaluated for severity of bacterial spot on leaves and stems (foliage) and fruits. The scale 0–11 developed by Horsfall and Barratt (1945) was used for the evaluation of severity of the disease on foliage. Symptomatic samples of foliage and fruits were collected for isolation of bacteria associated with the affected tissues (Table 1). Samples were stored at 4 °C until bacterial isolation was completed within 72 h post collection.

Isolation and maintenance of bacterial isolates. Bacteria were isolated from collected samples using the procedure reported by Schaad et al. (2001). Plant tissues were washed with tap water to remove soil and other particles, then 5- × 5-mm tissue sections containing lesions were cut and sections were surface-disinfested using 99% ethanol for 60 s. The sections were then washed three times (1 min each time) with sterile distilled water (SDW). Each section was inserted into a 15-mL glass test tube containing 10 mL of SDW and shaken by hand for 20 s to prepare a bacterial suspension. Then, 100 µL of the suspension was transferred onto nutrient agar (NA) and yeast dextrose calcium agar (YDC) media in petri plates. The plates were incubated at 28 °C for 72 h, at which time well-developed colonies on both culture media were subcultured onto NA. Isolates were stored in 4 mM sucrose and 20% glycerol (v:v) at –80 °C.

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Table 1. Plant samples collected from symptomatic foliage and fruits from different regions of Illinois during 2017–19.

Region	Number of farms (number of fields)	Number of samples collected	
		Foliage	Fruit
Northern[z]	3 (9)	59	18
Central[y]	3 (6)	28	3
Southern[x]	3 (12)	70	9

[z]Northern region included two farms in McHenry County and one farm in Kane County.
[y]Central region included two farms in Douglas County and one farm in Moultrie County.
[x]Southern region included three farms in Fayette County.

Identification of bacterial isolates. Isolated bacterial colonies were grown on YDC and then grouped based on their colony morphology and color. Consistent with Schaad et al. (2001), the colonies with yellow mucoid characteristics on YDC, which were suspected to be *Xanthomonas* spp., were subjected to polymerase chain reaction (PCR) using RST 65/69 (*hrpB7*) primers. Colonies with other colors (i.e., orange, white, pink) were subjected to PCR using different primers for amplifying 16S rRNA.

Species of the isolates with yellow mucoid colonies were identified based on amplicon sequencing from the PCR assay. Isolates were streaked onto NA and incubated at 28 °C for 72 h The *Xanthomonas*-specific primers RST 65 (5′-GTCGTCGTTACGG-CAAGGTGGTCG-3′) and RST 69 (5′-TCGCCCAGCGTCATCAGGCCATC-3′), which amplify a 420 bp fragment of *hrp*B7 (Obradovic et al., 2004), were used for PCR amplification. For each isolate, a 25-µL mixture containing *Taq* DNA polymerase (Omega Bio-Tek Inc., Norcross, GA), RST 65/69 primers, deoxyribonucleotide triphosphates (dNTPs), and a small number of bacterial cells (Leite et al., 1994) were used for the reaction. PCR amplification was performed using a ProFlex thermal cycler (Thermo Fisher Scientific, Waltham, MA), with initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 95 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 45 s; followed by a final extension at 72 °C for 5 min (Obradovic et al., 2004). Then, 5 µL of each PCR reaction was analyzed using gel electrophoresis with 1% agarose gel containing EZ-vision DNA dye (VWR Life Sciences, Radnor, PA), run at 100 V for 30 min, and visualized at 470 nm by the Azure Biosystems c400 imager (Dublin, CA).

The remaining PCR product was purified according to the manufacturer's recommendation using EXOSAP-IT (Thermo Fisher Scientific) and sent for Sanger sequencing at the DNA Services Laboratory, Roy J. Carver Biotechnology Center, University of Illinois. Sequences were analyzed using the BLASTN database from the National Center for Biotechnology Information (NCBI).

Pathogenicity tests. Pathogenicity of *Xanthomonas* isolates was conducted on four tomato cultivars (Brandywine, Dixie Red, Primo Red, and Red Deuce) in a greenhouse, using two isolates from each of the northern, central, and southern regions. Tomato seeds were sown in plastic pots (20 cm in diameter) filled with a growth medium mix of soil:-peat:perlite (1:1:1; v:v). The pots were placed in a greenhouse at 26 °C during day (14 h) and 24 °C at night (10 h). The bacterial isolates were grown on NA for 48 h, then colonies were washed with SDW, and a suspension with 5×10^8 CFU/mL (OD_{600} = 0.3) was prepared (Kyeon et al., 2016). Eight- to ten-week-old plants were inoculated by infiltrating 0.3 mL of the bacterial suspension into each leaflet using a needleless syringe. Three plants of each cultivar and four leaves of each plant were inoculated with each isolate. Control plants were infiltrated with SDW. The development of the symptoms was monitored and recorded at 3, 5, and 7 d post inoculation. Tissues from inoculated and control plants were processed for isolation of bacteria, as previously described.

Phylogenetic analysis. Isolates identified as *Xanthomonas* were used for the development of a phylogenetic tree using MLSA. The isolates were evaluated using the six housekeeping genes *fusA*, *gap-1*, *gltA*, *gyrB*, *lacF*, and *lepA* (Almeida et al., 2010). Gene fragments obtained from the Sanger sequencing center were compared with the following whole genome sequences found in NCBI: *X. gardneri* ATCC 19865 (NZ_AEQX00000000); *X. gardneri* ICMP7383 (NZ_CP018731.1); *X. perforans* 91-118 (NZ_CP019725, representative Florida group 1, Xp-FL1); *X. perforans* Xp3-15 (JZVG01, representative Florida group 2, Xp-FL2); *X. perforans* Xp4-20 (JZUZ01, representative Florida group 3, Xp-FL3); and *X. euvesicatoria* 85-10 (NC_007508.1). *Stenotrophomonas maltophilia* K279a (NC_010943.1) was used as the outgroup.

All the gene sequence fragments, either obtained via Sanger sequencing or extracted from whole genome sequences, were aligned using CLUSTALW within MEGA 10.0.5 (Kumar et al., 2018) and trimmed to the same length using the same starting position. For each isolate, the trimmed sequence for the six genes were concatenated for a total of 3092 nucleotides. All concatenated sequences were aligned, and the Akaike Information Criterion (AIC) within jModeltest 1.1 (Posada and Buckley, 2004) was used to select the nucleotide substitution model that best fit the aligned sequences. The Tamura 3-parameter model with gamma distribution and with invariant sites (T92 + G + I) was used for constructing the phylogenetic trees. The maximum likelihood tree was determined using the concatenated sequences with 1000 bootstrap samples. Genetic groups were defined as isolates having less than 10 nucleotide differences.

Identification of non-Xanthomonas isolates. Isolates that tested negative for *hrp*B7 amplification were further analyzed for genus identification and their pathogenicity on tomato. Identification of non-*Xanthomonas* isolates was achieved by using 16S rRNA sequencing. PCR amplification of 16S rRNA was performed using the primer set 27F (5′-AGAGTTTGATCMGGCTCAG-3′) and 1492 R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). PCR amplification, purification, and sequencing was performed as described above for *Xanthomonas* isolates, except for the PCR amplification conditions, which consisted of 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. Sequences were analyzed using the BLASTN database from the NCBI to assign each isolate to a particular genus. Isolates that did not have a match in BLASTN database were labeled as "unknown."

Two isolates of each of non-*Xanthomonas* bacteria, collected in 2018 and 2019, were tested for their pathogenicity on tomatoes in the greenhouse using the same method described above for *Xanthomonas* isolates. However, only two tomato cultivars, Red Deuce and Brandywine, were used with non-*Xanthomonas* bacteria.

Results

Incidence and severity of bacterial spot. Bacterial spot was observed in 26 of 27 fields visited during 2017–19. In 2017 and 2019, average disease severity on foliage was highest in southern Illinois, while in 2018 it was the highest in northern Illinois (Table 2). The highest range of disease severity (0% to 91%) was observed in northern Illinois in 2019, and the lowest range was observed in central Illinois in 2017 (2% to 4.5%). Overall, disease severity of foliage was highest in the 2019 season (Table 2). Incidence of symptomatic fruit was generally low, with the overall highest incidence occurring in 2019 and the lowest in 2017 (Table 3).

Identification of Xanthomonas spp. A total of 266 isolates that produced the 420 bp *hrp*B7 amplicon in PCR tests were identified as *Xanthomonas* spp. Of the 266 isolates, 221 were obtained from foliar tissues and 45 from fruits (Table 4). Of 91 isolates collected from the northern region, 13 and 78 isolates were *X. perforans* and *X. gardneri,* respectively. In contrast, of the 139 isolates collected from the southern region, 101 and 38 isolates were identified as *X. perforans* and *X. gardneri,* respectively. Among the 36 isolates collected from the central region, 19 and 17 isolates were *X. perforans* and *X. gardneri,* respectively (Table 4).

Pathogenicity test. All tested *Xanthomonas* isolates produced symptoms of bacterial spot as observed in the fields. Bacteria were re-isolated from the infiltrated leaves and produced the *hrp*B7 gene fragment in PCR assays. Plants infiltrated with SDW did not develop any lesions, and no bacteria were isolated from tissues infiltrated with SDW.

Fig. 1. Stars indicate Illinois counties where commercial tomato fields were surveyed for the occurrence of bacterial spot disease. Kane and McHenry, Douglas and Moultrie, and Fayette counties are reported as northern, central, and southern regions, respectively.

Multilocus sequence analysis of isolated Xanthomonas spp. X. perforans Xp-IL1 was the most prevalent (90 isolates) among the genetic groups of *X. perforans* (Fig. 1) and was like the reference strain *X. perforans* Xp-FL1 (Fig. 2). *X. perforans* Xp-IL1 was collected from seven of the nine farms. *X. perforans* Xp-IL2 (36 isolates) was found only in a single farm in the southern region and was like the reference strain *X. perforans* Xp-FL2. *X. gardneri* Xg-IL was the most prevalent (119 isolates) isolate and was like the reference isolate *X. gardneri* ATCC 19865 (Fig. 2). *X. gardneri* Xg-IL isolates were found in all nine farms across all three regions. Tissue samples infected with both *X. perforans* and *X. gardneri* were collected from six of nine farms. Of the original 266 isolates, 7 *X. perforans* and 14 *X. gardneri* isolates were not fully sequenced and were removed from the analysis.

Identification of non-Xanthomonas bacteria. A total of 412 bacterial isolates were collected from symptomatic tomato tissues that did not produce yellow colonies characteristic of *Xanthomonas* on YDC. Furthermore, none of these colonies produced the 420 bp amplicon in the PCR assay with RST 65/69 primers, nor did they cause symptoms in infiltrated tomato leaves. Most of these non-*Xanthomonas* isolates belonged to nine bacterial genera, including *Agrobacterium*, *Bacillus*, *Curtobacterium*, *Microbacterium*, *Paenibacillus*, *Pantoea*, *Psuedomonas*, *Rhizobium*, and *Stenotrophomonas* (Table 5). Most identified isolates were *Microbacterium*, *Pantoea*, and *Pseudomonas* spp.

Discussion

This was the first survey of Illinois fields to assess the severity and identify the causal agents of bacterial spot disease on tomato plants and fruits. *X. perforans* and *X. gardneri* were identified as the incitants of the disease in Illinois. *X. perforans* was more prevalent in the southern region, while *X. gardneri* was the main species found in the northern region. In central Illinois, the distribution of *X. perforans* and *X. gardneri* was roughly equal at 55% and 45%, respectively. A previous study conducted in Ohio showed that *X. perforans* and *X. gardneri* were both present in tomato production fields (Ma et al., 2011), while other studies conducted in cooler climate regions have reported *X. gardneri* as the dominant species (Araujo et al., 2010; Cuppels et al., 2006; Kim et al., 2010). The findings in our study agree with these reports—that the prevalence of *X. gardneri* in northern Illinois is likely related to cooler weather conditions in this region. In addition, the results from our study showed that the populations of *X. perforans* in Illinois are genetically like those found in Florida, which has a warmer climate (Potnis et al., 2011). Moreover, a report from Brazil (Araujo et al., 2017), a place with warmer conditions, shows widespread distribution of *X. performance* and a limited presence of *X. gardneri,* which is like the findings in our study.

Multilocus sequence analysis revealed endemic populations of *X. gardneri* and *X. perforans* throughout Illinois. The widespread occurrence of genetically similar isolates likely originated from recurring infections caused by infected plant materials in previous years that subsequently spread between nearby fields, or via contaminated materials shared between production sites. The presence of multiple genetic groups of *X. perforans* has been reported in various tomato-growing regions such as North Carolina and Florida (Adhikari et al., 2019; Timilsina et al., 2015, 2019). About 71% of *X. perforans* isolates in our study (*X. perforans* Xp-IL1) grouped with

Table 2. Severity of bacterial spot symptoms on foliage in commercial tomato fields located in different regions of Illinois.

	Disease severity (%)[z]								
	2017			2018			2019		
Region	Lowest	Highest	Avg[y]	Lowest	Highest	Avg[y]	Lowest	Highest	Avg[y]
Northern	4.5	18.5	12	37.5	91	64	0	91	46
Central	2	4.5	3	9	18.5	10	4.5	62.5	55
Southern	9	37.5	20	9	81.5	50	37.5	91	70

[z]Percent affected foliage one week before the final harvest.
[y]Average disease severity.

Table 3. Incidence of tomato fruits with bacterial spot symptoms in commercial fields in Illinois.

	Symptomatic fruits (%)[z]								
	2017			2018			2019		
Region	Lowest	Highest	Avg[y]	Lowest	Highest	Avg[y]	Lowest	Highest	Avg[y]
Northern	0	9	4.5	0	25	12.5	0	25	12.5
Central	0	0	0	0	0	0	0	30	15
Southern	0	4	2	0	20	10	0	4	2

[z]Percent symptomatic fruits observed during the growing season.
[y]Average incidence of fruits with bacterial spot symptoms during the growing season.

Table 4. Number of *Xanthomonas* isolates collected from different regions of Illinois.

	Foliage[z]		Fruit	
Regions	*X. perforans*	*X. gardneri*	*X. perforans*	*X. gardneri*
Northern	9	54	4	24
Central	17	13	2	4
Southern	94	34	7	4

[z]Isolated from leaves and stems.

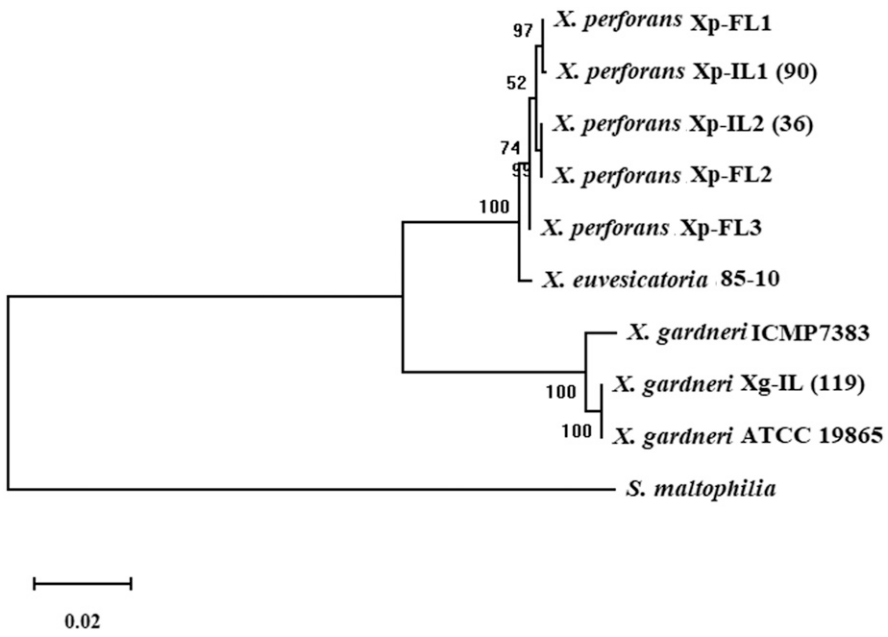

Fig. 2. Clustering of isolates of *Xanthomonas perforans* and *X. gardneri* from commercial tomato fields in Illinois during 2017–19. Multilocus sequence analysis of six housekeeping genes (*fusA*, *gap-1*, *gltA*, *gyrB*, *lepA*, and *lacF*) were used to group the isolates. Isolates of *X. perforans* Xp-IL1, *X. perforans* Xp-IL2, and *X. gardneri* Xg-IL were from Illinois; *X. perforans* Xp-FL1, *X. perforans* Xp-FL2, *X. perforans* Xp-FL3, *X. euvesicatoria* 85-10, *X. gardneri* ICMP7383, and *X. gardneri* ATCC 19865 are reference isolates in the NCBI; and *S. maltophilia* = *Stenotrophomonas maltophilia*. Numbers inside the parentheses indicate the numbers of the isolates classified within that particular genetic group, with Illinois genetic groups defined as having fewer than 10 different nucleotides over the total 3092 nucleotides. The scale bar represents the number of substitutions per site, and values on the branches indicate Bayesian posterior probabilities expressed as the percentage of trees based on 1000 bootstrap replicates.

X. perforans Xp-FL1 (a reference isolate) suggested endemic dispersal of this genetic group. About 29% of *X. perforans* isolates (*X. perforans* Xp-IL2) were grouped with *X. perforans* Xp-FL2 strains (Timilsina et al., 2015); however, these were isolated from a single farm, which suggests initial infection from different source that, likely over a period of some years, led to the endemic presence of Xp-IL2 isolates at this particular location. Previous studies found that a single genetic strain of *X. gardneri* has been distributed around the world (Kebede et al., 2014; Timilsina et al., 2015). Consistent with these findings, the *X. gardneri* Xg-IL isolates from Illinois tomato fields were identical to isolates from this genetic group.

Table 5. Non-*Xanthomonas* bacteria isolated from symptomatic tomato foliage and fruits.

	Number of isolates		
Bacterial genus	2017	2018	2019
Agrobacterium	0	5	5
Bacillus	4	9	5
Curtobacterium	3	7	6
Microbacterium	10	82	15
Paenibacillus	12	1	5
Pantoea	0	18	35
Pseudomonas	0	56	35
Rhizobium	0	7	4
Stenotrophomonas	0	6	15
Unknown	12	75	20
Total	41	266	145

Identification of pathogens has been a driving factor in the development of resistant cultivars for disease management. Because all tomato cultivars grown at the nine Illinois commercial farms included in this study were found to be susceptible to bacterial spot disease, and with evidence of endemic spread of the pathogens across the state, future research should focus on developing more effective management strategies, including breeding for resistance, inducing resistance by using chemicals or biopesticides, cultural practices, and chemical use.

In this study, *Xanthomonas* and non-*Xanthomonas* bacteria were isolated from symptomatic tomato foliage and fruits. All *Xanthomonas* isolates tested were pathogenic on tomato leaves, while none of the non-*Xanthomonas* isolates caused any symptoms. We concluded that the non-*Xanthomonas* bacteria isolated from tomato foliage and fruits are likely nonpathogenic. However, our findings do not preclude the possibility that some of these bacteria may act synergistically with *Xanthomonas* to cause greater disease symptoms, or that some may be antagonistic to the *Xanthomonas* bacteria. Future research would be needed to evaluate whether co-occurrence of these bacteria during tomato infection by *Xanthomonas* affects the development of the disease symptoms.

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